Docket No. 55322 (71699)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	C. LeVisage, et al.
Serial No.:	09/975,565
Filed:	October 11, 2001
For:	POLYMER CONTROLLED DELIVERY OF A THERAPEUTIC AGENT
Examiner:	B. M. Fubara
Art Unit:	1615

Mail Stop: Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

DECLARATION UNDER 37 C.F.R. 1.132

I, Kam Leong, a citizen of the United States of America residing at 10242 Breconshire Road, Ellicott City, MD 21042, hereby declare as follows:

1. I am a co-inventor of the subject matter described and claimed in the patent application U.S.S.N. 09975,565, filed on October 11, 2001 and otherwise identified above.

2. I have read and understood the Office Action dated December 29, 2004 and the references cited in the Office Action in the above case.

3. The following experiments were conducted by me or under my supervision, to compare the therapeutic effect of pharmaceutical compositions of a therapeutic agent

encapsulated in a polymer support wherein the composition comprises microparticles having a mean particle size of 1.0-100 μm against pharmaceutical compositions comprising nanoparticles having a mean particle size of < 500 nm.

4. Comparison uptake from the bladder for nanoparticles and microparticles composed of poly(phosphoester) poly(D,L-lactide-*co*-ethyl phosphate) {referred to herein as "P(DAPG-EOP)"} having encapsulated LacZ DNA. Experiments originally disclosed in 09/972,725, filed October 5, 2001, now issued as U.S. Patent 6,797,704.

Experiment 4.1

Microspheres comprising a biocompatible polymer and a therapeutic agent were prepared via variations of a water/oil/water (w/o/w) double-emulsion technique (FIG. 1). An aqueous solution of LacZ DNA (2 mg) dissolved in 200 μL water was added to P(DAPG-EOP) (100 mg) dissolved in 1 mL methylene chloride and sonicated to form the primary water/oil (w/o) emulsion. This mixture was added to 5 mL of 5% NaCl, 1% poly(vinyl alcohol) (PVA) and either sonicated or vortexed to form the secondary (w/o/w) emulsion. This was then added to 40 mL of 5% NaCl, 0.3% PVA, and stirred for 3 hours at room temperature to evaporate the methylene chloride. The microspheres were washed 3 times with water and lyophilized for storage.

Experiment 4.2

Microspheres containing LacZ were prepared in two size ranges: one had a median size of 600 nm ("nanospheres"), while the second ranged in size from 1 - 15 μm, with a median number size near 5 μm ("microspheres"). In order to provide reliable tracking, the dye needed to remain associated with the polymer and continue to be fluorescent at low pH, as the nanospheres or microspheres were subjected to the slightly acidic environment of the urinary bladder. Experiments showed that the hydrophobic dye remain firmly associated with the polymer for at least one week in water at 4°C, because no dye was found in the supernatant following centrifugation for 5 minutes at 10,000 RPM. Incubation of the dye with 10N HCl

did not alter the dye's fluorescent properties. Furthermore, the dye remained associated with the polymer and retained its fluorescent properties when incubated with naïve mouse urine in vitro for extended periods at room temperature.

Experiment 4.3

For bladder instillation, animals were rendered unconscious with Metofane (Schering-Plough, NJ) and laid on their back. The mouse clitoris was gently held with forceps, while the urethra was catheterized until the anterior wall of the bladder was encountered (FIG. 2). The bladder was palpated to remove any urine. Eighty μL of microsphere or nanosphere solution containing approximately 1.25 mg microspheres or nanospheres (25 μg LacZ DNA) was instilled, followed by removal of the catheter. The mice were observed until full consciousness returned.

Experiment 4.4

Microparticles or nanoparticles of P(DAPG-EOP) comprising Nile Red dye were instilled via a catheter into the bladder of a plurality of mice. Urine was collected over a period of four hours and the concentration of dye including both free dye molecules and dye dispersed in microparticles was measured by fluoroscopy. For small microparticles, e.g., nanospheres, dye was observed in the urine only during the first 30 minutes. Dye was observed in the urine of mice instilled with larger micropartices, e.g., mean particle size of about 5 μm, for up to three hours after instillation and the concentration of dye was up to 16 fold greater than in the urine of mice instilled with nanospheres. The dye concentration in urine for mice instilled with larger microparticles was measured fluorometrically (FIG. 3). More than 90% of the administered dose delivered by nanospheres is transported to the lymph node within three hours of instillation.

Experiment 4.5

Standard confocal microscopic analysis of organ sections of mice sacrificed at 30

minutes, 90 minutes, 3 h and 48 hours after bladder instillation was performed. For the nanospheres, after 30 minutes, nanospheres were visible in the bladder lumen and along the mucosal cells (FIG 4A). No nanospheres were observed in the bladder at any of the later time points. This was consistent with the findings of the Experiment 4.4, where few nanospheres were found in the urine. The nanospheres were not found in lung, spleen, or liver sections at any time point, but were seen in the lymphatics at 90 minutes and in lymph nodes at the 3-hour time point (FIG. 4B).

In contrast, the microspheres (5 μm) were not observed outside the bladder by standard confocal microscopic analysis of organ sections of mice sacrificed at 30 minutes, 90 minutes, 3 h, or 48 h after bladder instillation.

Example 4.6

To examine the mechanism of uptake of the nanospheres from the bladder lumen, P(DAPG-EOP) nanospheres containing DNA were instilled into the bladders of female Balb/c mice. These mice that were sacrificed at either 7 or 20 minutes and their bladders prepared for TEM (500 nm sections). FIG. 5A-C provides TEM images of mouse bladder with microparticles contained therein.

5. Installation of pharmaceutical compositions of microspheres comprising a poly(methylidene malonate 2.1.2 (referred to herein as PPM 2.1.2) and paclitaxel adhere to the mucosa of the bladder without being washed out in the urine or absorption into other tissues or organs.

Experiment 5.1: Preparation of microparticles with encapsulated paclitaxel.

Paclitaxel-encapsulated PMM 2.1.2 microparticles were prepared by a modified solvent evaporation technique previously described (Bru-Magniez, PCT WO 99/55309). Sulfur dioxide free 1-ethoxycarbonyl-1-ethoxycarbonylmethyleneoxy carbonyl ethene was first dispersed in acetone (1% v/v) and sodium hydroxide (0.1 M) was added to the magnetically stirred acetone dispersion until the sodium hydroxide concentration in acetone was 1%. Polymerization

occurred after 5 minutes of stirring and the polymer was recovered after evaporation of the acetone under vacuum. An organic solution of polymer (50 mg in 1.5 mL of ethyl acetate) containing 2.5 mg of paclitaxel (Sigma Chemicals, Inc.) was poured into 15 mL of an aqueous solution of poly(vinylalcohol) (88% hydrolyzed, from Polysciences) (2% w/v) and the emulsification process was conducted during 5 minutes (Polytron PT 1200). The resulting emulsion was then stirred at room temperature during at least 4 hours, until complete evaporation of the ethyl acetate. Hardened microparticles were then isolated by centrifugation, washed 3 times with distilled water then stored at 4°C. The average particle size of the PMM 2.1.2/paclitaxel microparticles is about 2 μm in diameter.

Experiment 5.2. Distribution of microparticles after administration to mice.

Fluorescent microparticles, prepared with nile red, were administered to remail Balb-c mice (8 weeks old) as a single dose intravesically (50 μL). Animals were sacrificed after 30 minutes, 3 and 48 hours. The bladder was excised, fixed in paraformaldehyde 3% during 2 hours then embedded on OCT and frozen. Tissue cryosections (20 μm) (Cryostat) were observed by using a confocal microscope (Zweiss Axiovert 100) with filters for selective FITC excitation (detection of green autofluorescence of the tissues) and selective nile red excitation (for detection of microparticles). Particles were also identified on Hematoxylin and Rosin stained sections. Non-fluorescent particles were also instillated to mice in the same conditions and scanning electron microscopy was preformed on bladder sections to localize particles.

An in vivo observation of microparticles localization in the bladder was performed with scanning electron microscopy. Thirty minutes after the intravesical administration of microparticles encapsulating paclitaxel into mice, particles were observed mainly in the lumen of the bladder (FIG. 6 {Figure 4 of the specification of 09/975,565}).

With scanning electron microscopy, we were able to observe localization of the microparticles at the surface of the mucosa. Surprisingly, particles remained attached to the mucosa after 3 hours and some particles could still be observed by standard confocal

microscopic analysis attached to the mucosa after 48 hours. Microparticles were not observed in other tissues and organs of the patient at 3 hours or 48 hours post administration by standard confocal microscopic analysis.

Thus, these particles can deliver a bioactive molecule specifically to a targeted position such as a bladder mucosa and that a controlled release can be achieved.

6. The results of Experiment 4 illustrate the size dependent biological uptake of microparticles versus nanoparticles. Nanoparticles of P(DAPG-EOP) having encapsulated LacZ DNA and a median size of 600 nm localized to the lymph nodes within 3 hours of administration to the bladder. None of the nanoparticles were observed in the bladder after 30 minutes. In contrast, microparticles of P(DAPG-EOP) having encapsulated LacZ DNA with a range of particle sizes from 1 - 15 μm and a median particle size of about 5 μm were retained in the bladder for at least three hours with negligible transport to other tissues or organs.

The uptake behavior of PMM 2.1.2 polymer microparticles having a particle size of about 2 μm is similar to the 5 μm P(DAPG-EOP) microparticles. That is, 2 μm PMM 2.1.2 polymer microparticles administered to the bladder adhered to the mucosa of the bladder

From these results and based on my understanding of drug delivery systems, including microparticle polymer drug delivery systems, I reasonably conclude that small nanoparticles, e.g., <500 nm, composed of PMM 2.1.2 will likely be transferred from the bladder to other tissues or organs in a patient after intrabladder administration.

This discovery was not obvious to one of ordinary skill in the art, even in view of the cited reference. Therefore, we (the inventors of the present invention) declare that the present invention concerning (1) pharmaceutical compositions comprising a microphere having a particle size of between 1-100 μm composed of PPM 2.1.2 and a therapeutic agent; and (2) methods of using said pharmaceutical compositions for the treatment of urological disorders is inventive and non-obvious.

7. I, the undersigned Kam Leong, further declare that all statements made herein of my own knowledge are true and that all statements made upon information and belief are believed

to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under Section 101 of Title 18 of the United States Code and that such willful false statement may jeopardize the validity of the above identified application or any patent issuing thereon.

5

By: Kam Leong

Kam Leong

Date: May 31, 2005

10

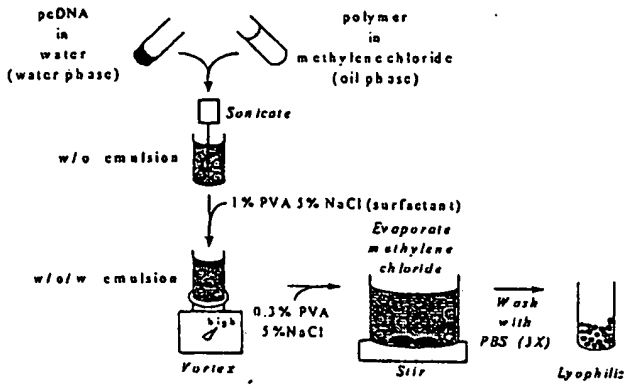

FIG.1

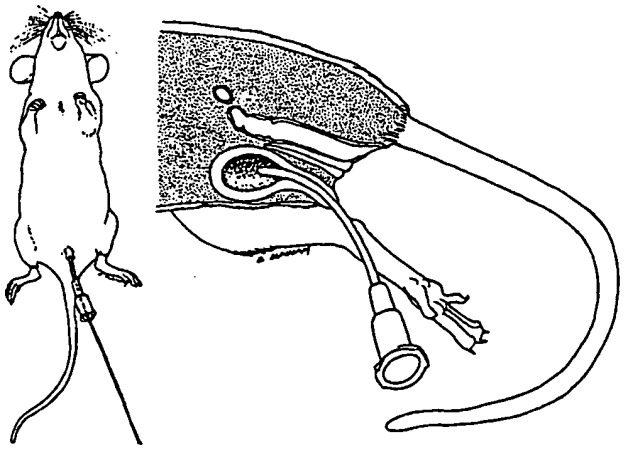

FIG. 2

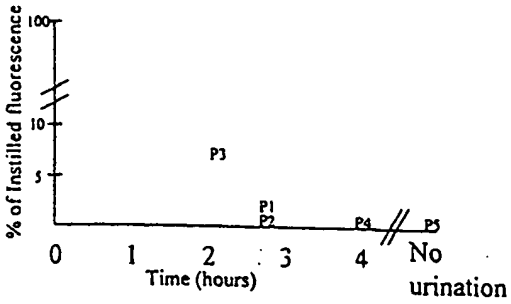

FIG. 3

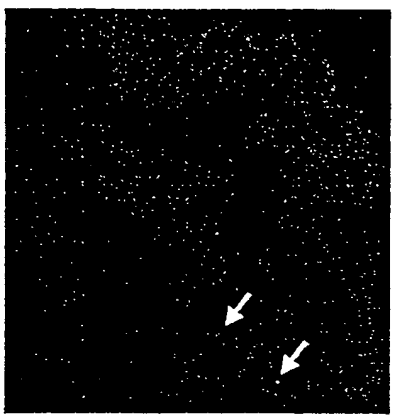

FIG. 4A

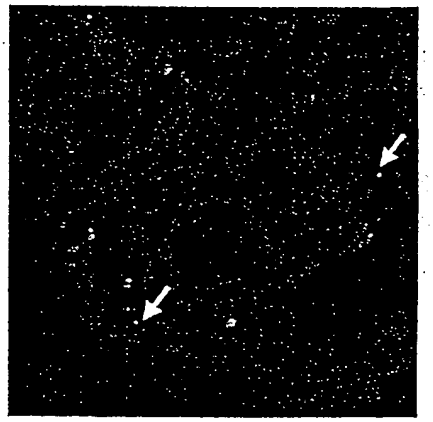

FIG. 4B

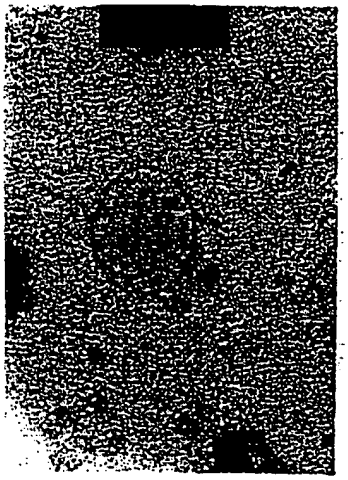

FIG. 5A

FIG. 5B

FIG. 5C

5

10